Potato Spindle Tuber Disease: Procedures for the Detection of Viroid RNA and Certification of Disease-Free Potato Tubers

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ABSTRACT

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Detection of viroid RNA in small quantities of potato spindle tuber viroid-infected potato and tomato terminal tip tissue has been used for rapid and reliable diagnosis of the disease. The method involved the extraction of cellular nucleic acids followed by electrophoretic analysis of the extracts on 5% polyacrylamide gels. Initial inoculation of tomato plants with extracts from potato terminal tip tissue and subsequent analysis of the inoculated tomatoes by the gel method allowed detection of one infected plant in a 250-plant test. Application of these procedures to a routine potato-indexing program is discussed in relation to the possibility of producing seed potatoes certified to be free of this disease.

Potato spindle tuber viroid (PSTV) presents some problems to certified seed potato producers. The disease is easily transmitted in the field by contact and it is often difficult to diagnose because symptoms may be obscure or lacking. Reluctance to import potatoes not certified to be free of this disease into areas where it is not known to occur is understandable. This might also be of concern to citrus growers, considering recent evidence which indicates the similarity of PSTV to the citrus exocortis pathogen (8).

Spindle tuber has been shown to be incited by a low-molecular-weight RNA termed a "viroid" by Diener (2). Semancik and Weathers (9) first reported the isolation and detection of a similar viroid RNA from citrus exocortis-infected plants using polyacrylamide gel electrophoresis. Morris and Wright (5) subsequently published a diagnostic procedure for PSTV using nucleic acid extraction and polyacrylamide gel electrophoresis. Although impractical for testing large numbers of samples because of procedural complexity, the test proved more rapid and reliable than existing methods of assay (4, 10). It has been adopted for rapid confirmation of suspected outbreaks of the disease and for pre-elite seed testing in British Columbia and New Brunswick. In this report we describe a modified procedure which permits more rapid and efficient testing and include a second test adaptable for larger-scale indexing. We propose a certification program using these methods which should be an aid in eliminating this disease from potato seed stock in North America.

MATERIALS AND METHODS

Severe and mild strains of PSTV were isolated locally or obtained from N. S. Wright, Agriculture Canada, Vancouver. The viroids were maintained in potato *(Solanum tuberosum* L. 'Kennebec') grown from infected tubers and tomato (*Lycopersicon esculentum* Mill. 'Rutgers') inoculated on the cotyledons before the first true leaves were expanded. The plants were grown in the greenhouse above 25 C without supplemental lights. Better growth and symptom development was obtained by maintaining infected plants in a growth chamber at 27-30 C with a 16-hour daylength.

Electrophoretic separation of the nucleic acid preparations was performed on 5% polyacrylamide gels in 0.04 M tris, 0.02 M sodium acetate, 0.001 M disodium EDTA pH 7.2 (1). Electrophoresis was performed in cylindrical gels (0.6 × 9 cm) at 12-15 C in a Biorad Model 150 Cell equipped with a water jacket. The gels were prerun for 30 minutes at 75 V and then the nucleic acid solution (0.2 ml of nucleic acid in distilled water with a drop of saturated sucrose and 0.05% bromophenol blue tracer dye) was applied to the top of each gel. The gels were electrophoresed for about 4 hours until the tracer dye was 1 cm from the bottom of the gel (100 V, 5-7 mA per gel). The gels then were removed from the tubes and stained overnight with 0.1% toluidine blue O in water. The gels were destained with tap water and stored in 0.01% sodium azide.

Quantitative determination of the RNA bands in the gels was estimated by scanning unstained gels at 280 nm (preferred to 254 nm because background due to acrylamide impurities is reduced) or stained gels at 590 nm in Varian Model 635 spectrophotometer equipped

with a linear transport device. The concentration of the RNA species was determined by comparing the area of the peaks to a standard curve prepared from known concentrations of 5S RNA.

PROCEDURES AND RESULTS

Different nucleic acid isolation procedures were evaluated using a range of extraction buffers, detergents, and protein denaturants (6). The following procedures consistently proved to be the most effective for extracting the viroid RNA fraction and they were adopted for routine isolation of viroid RNA from small quantities of infected potato and tomato terminal tip tissue. It is essentially a shorter version of the previously published method of nucleic acid extraction (5). The amount of tissue processed and the volumes of reagents used in the extraction have been reduced five-fold and the number of manipulative and centrifugation steps also have been reduced by one-half, thus decreasing the time required to perform the test. Samples extracted on the first day can be tested on gels the next day. The procedural steps given below are for the extraction of 1 g of tissue; the quantities of reagents should be increased or decreased in proportion to the amount of tissue to be processed.

Procedures.—*Step 1.*—Grind 1 g of terminal tip tissue in 0.5 ml of extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, 1.0% sodium lauryl sulfate adjusted to pH 9.5 with 5 M NaOH), 1 drop of mercaptoethanol, 2 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline and 2 ml of chloroform-pentanol (25:1, v/v). Extraction is accomplished by mixing all the reagents with the tissue and homogenizing the mixture for a few seconds in a VirTis or Polytron homogenizer at room temperature. Alternatively, the tissue may be extracted in a small mortar and pestle by crushing it in extraction buffer and phenol, and then stirring in the chloroform-pentanol to form an emulsion.

Step 2.—Centrifuge the homogenate at 10,000 rpm (12,100 *g*, 4 C) for 15 minutes in a Sorvall RC2 centrifuge in an SS 34 rotor. Draw off the clear upper aqueous phase (about 1 ml) with a pasteur pipette after centrifugation.

Step 3.—Add one-fifth volumes of 10 M LiCl to the supernatant to make a final concentration of 2 M LiCl in the solution (0.25 ml of 10 M LiCl per ml of supernatant). A heavy white precipitate will form. Incubate this solution on ice for 2 hours.

Step 4.—Centrifuge the solution at 8,000 rpm (7,719 *g*, 4 C) for 15 minutes. Decant the clear supernatant solution and dialyze it exhaustively against cold distilled water overnight at 4 C.

Step 5.—The samples are ready for electrophoresis after dialysis. Apply 0.5 ml of solution to the gel, approximately one-third to one-half of the sample. The processing of larger quantities of tissue needed for multiple tests and tomato indexing will require a final ethanol concentration step after dialysis. Two volumes of 95% ethanol and a few drops of 4 M sodium acetate are added to the solution and the nucleic acid is allowed to precipitate in the freezer for 1 hour. The precipitate is collected by centrifugation (8,000 rpm for 15 minutes), the ethanol is discarded, and the nucleic acid pellet is allowed to dry with the tube inverted to permit the ethanol to evaporate. The precipitate is then resuspended in 0.2 ml of distilled water and stored frozed at −20 C until it is electrophoresed. The precipitate will be barely visible after drying and should resuspend immediately and produce a clear solution if the dialysis has been sufficient. The dialysis step may have to be repeated for a few hours if the resuspended precipitate is cloudy.

The results of a typical extraction of infected and healthy potato terminal tip tissue are illustrated in Fig. 1-A. Single growing tips weighing 0.5 g were extracted in 0.25 ml of extraction buffer, 1 ml of phenol, and 1 ml of chloroform-pentanol. The final volume of each sample after dialysis was 0.5 ml and the whole sample was applied to gels without concentration by ethanol precipitation. The disease-specific RNA species is readily observed in the stained polyacrylamide gel containing nucleic acid from the infected tissue but not in similar gels prepared from healthy tissue. The disease-specific RNA band is diagnostic for PSTV infection and therefore permits detection of the essentially symptomless infections of the mild strains in potato and tomato plants (5).

The sensitivity of detection and the quality of the RNA extracted was comparable to that described previously (5). A comparison of the nucleic acids extracted from 1 g of young potato tissue by the two methods is illustrated in Fig. 1-B. No consistent differences in the concentration of the RNA bands in the gels containing RNA extracted by the previously published procedure (5) or the shorter procedure were detected by gel scanning.

The type and quantity of tissue required for successful

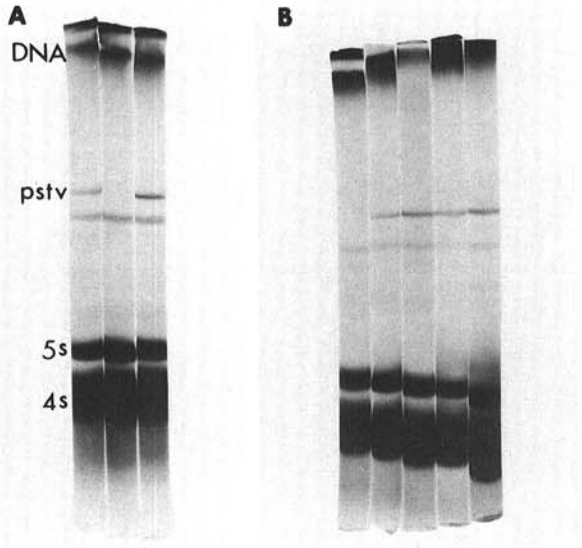

Fig. 1-(A, B). Polyacrylamide gels (5%) showing fractionated RNA extracted from 0.5-g samples of young potato tissue. The RNA was electrophoresed at 100 V, 6 mA/gel for 4 hours at 12 C and stained in 0.1% toluidine blue O. The gels contain nucleic acid extracted from (left to right): **A)** infected, healthy and infected tissue. **B)** healthy and infected (gels 2 and 3) tissue by the short procedure and infected (gels 4 and 5) tissue by the previously published method (5).

detection of PSTV-specific RNA was examined (Fig. 2). Potato spindle tuber viroid was readily detected in all types of infected tissue including fresh or frozen potato tissue, young tomato tissue from plants infected for 30 days at 30 C, etiolated potato tuber sprouts, and dormant tuber tissue. The young growing terminal tips of infected potatoes were the best source of viroid, allowing detection in as little as 0.2 g of tissue (Fig. 2-B). The optimal quantity of nucleic acid was obtained from 1 g of tissue which yielded about 200 μg of LiCl-soluble nucleic acid. The gels would, however, accommodate the nucleic acid extracted from up to 2 g of this type of tissue without appreciable loss of resolution of the RNA bands. A comparison of the concentration of the RNA species in the gels by scanning revealed that the quantity of RNA extracted was proportional to the amount of tissue processed. In addition, recovery was not appreciably improved by re-extracting the organic phase or by using a larger volume of buffer in the initial extraction. This is an indication that the recovery of nucleic acid was somewhat quantitative.

The ability to detect PSTV in small quantities of tissue allowed for testing of up to five plants in a single mixed sample. Gels containing nucleic acid extracted from 1-g samples consisting of five terminal growing tips weighing 0.2 g each are illustrated in Fig. 3. The method readily detected one infected plant in five when mixed samples were indexed. Successful detection of one infected tip in ten using 2-g samples was possible, but the results were not always consistent and gel anomalies sometimes resulted due to the higher quantity of nucleic acid in such samples.

Infectivity studies.—The disease-specific RNA observed in nucleic acid preparations from infected tissue is the infectious viroid. Identification of the infectious disease-specific RNA for the citrus exocortis agent in 5% gels (8) and for PSTV in 20% gels (2) has been reported. A typical scanning profile of the nucleic acids isolated from infected potato tissue and separated on a 5% polyacrylamide gel is shown in Fig. 4. The gel was scanned at 280 nm after electrophoresis and cut into 0.4-cm fractions using a Biorad Model 190 gel slicer. The gel slices were crushed in 1 ml of buffer (0.05 M glycine, 0.03 M K_2HPO_4, pH 9.2) to elute the RNA. The eluted fractions were then inoculated, at a dilution of 10^{-3}, to tomato plants grown at 30 C and symptoms were recorded 30 days after inoculation. It is apparent that the bulk of the infectivity is associated with the disease-specific RNA species and it is therefore concluded that the RNA species is the infectious viroid.

Typical scanning profiles of gels stained with toluidine blue O containing RNA extracted from healthy and infected tissue, treated with 20 μg/ml of DNase for 1 hour at 36 C to remove the DNA, are illustrated in Fig. 5. The concentration of PSTV-RNA in the nonfractionated preparation (Fig. 5, profile B) was estimated at 1.4 μg/ml from the gel scan. The PSTV-RNA shown in Fig. 5, profile C was purified by gel electrophoresis and had a concentration of 1.6 μg/ml. These two preparations were

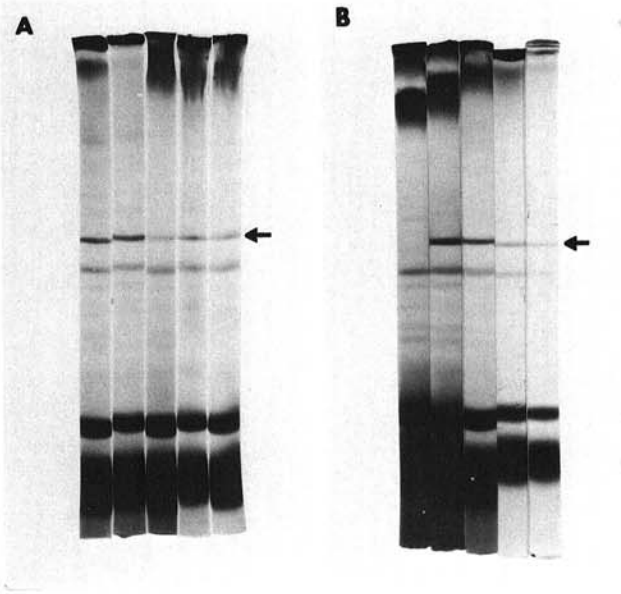

Fig. 2-(A, B). Polyacrylamide gels (5%) showing fractionated RNA extracted from different types and quantities of tissue. The RNA was electrophoresed at 100 V, 6 mA/gel for 4 hours at 12 C and stained in 0.1% toluidine blue O. The arrow locates the position of the potato spindle tuber viroid RNA. The gels contain nucleic acid extracted from (left to right): **A)** One gram of fresh young potato terminal tip tissue, frozen potato tissue, young tomato tissue, etiolated potato tuber sprouts and 2 g of dormant tuber tissue. **B)** Young potato terminal tip tissue consisting of 2 g of healthy- and 2.0 g, 1.0 g, 0.5 g, and 0.2 g of infected plants.

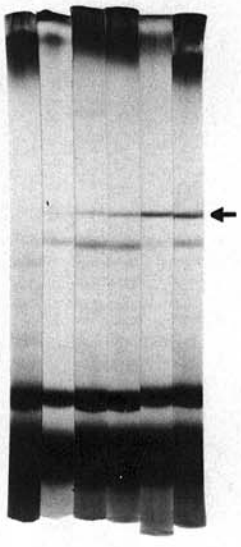

Fig. 3. Polyacrylamide gels (5%) showing fractionated RNA from 1-g samples consisting of five potato tips weighing 0.2 g each. The RNA was electrophoresed at 100 V, 6 mA/gel for 4 hours at 12 C and stained in 0.1% toluidine blue O. The arrow locates the position of the potato spindle tuber viroid RNA. The gels contain nucleic acid extracted from (left to right) samples containing 0, 1, 2, 3, 4, and 5 infected terminal tips.

serially diluted and inoculated to tomato plants. Infection on 50% of the inoculated tomatoes was recorded at dilutions of 10^{-7} to 10^{-9} for both preparations. Systemic assay on tomato plants was not a very accurate method for detecting small differences in viroid RNA concentrations, but the experiment indicated that almost all of the PSTV infectivity was associated with the purified disease-specific RNA.

Concentration of viroid RNA.—The concentration of PSTV-RNA in potato plants grown from tubers infected with a mild strain of the viroid was highest in the young growing tips and lower in mature leaves and stems (5). Examination of tomato plants (80 days after inoculation) and potato plants (grown from infected tubers) infected with severe and mild strains of PSTV showed essentially the same trend (Table 1). The lower concentration of viroid in the older tissue was essentially proportional to the lower overall nucleic acid content of such tissue (on a gram fresh weight basis) compared to younger actively growing tissue. Temperature had a pronounced effect on the concentration of PSTV isolated from tissue. Plants were grown under controlled conditions at 25 C or 30 C with a 16-hour daylength. The concentration of PSTV was consistently higher in different tissues of plants grown at 30 C compared to plants grown at 25 C. The total RNA content of comparable tissues grown at the two temperatures was about the same. This indicates that PSTV synthesis was preferentially affected by the higher growing temperature.

The content of PSTV in the growing tips of potatoes at intervals after planting of infected tubers in the greenhouse at 25-30 C was monitored for the two strains of PSTV (Fig. 6). The concentration of viroid was

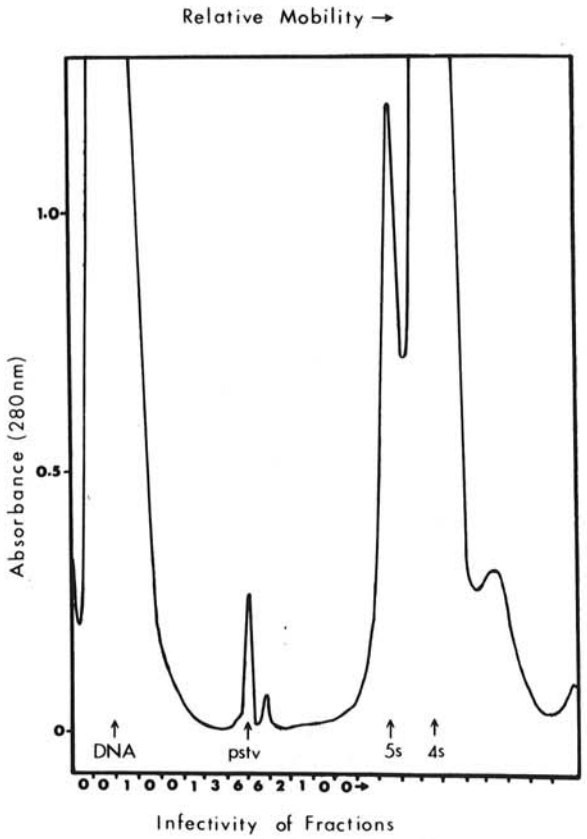

Fig. 4. Scanning profile of an unstained polyacrylamide gel (5%) containing nucleic acid extracted from infected potato tissue. The RNA was electrophoresed at 100 V, 6 mA/gel for 4 hours at 12 C and the gel then was scanned at 280 nm. The numbers on the lower axis indicate the number of tomato plants infected out of six inoculated with nucleic acid eluted from each gel fraction.

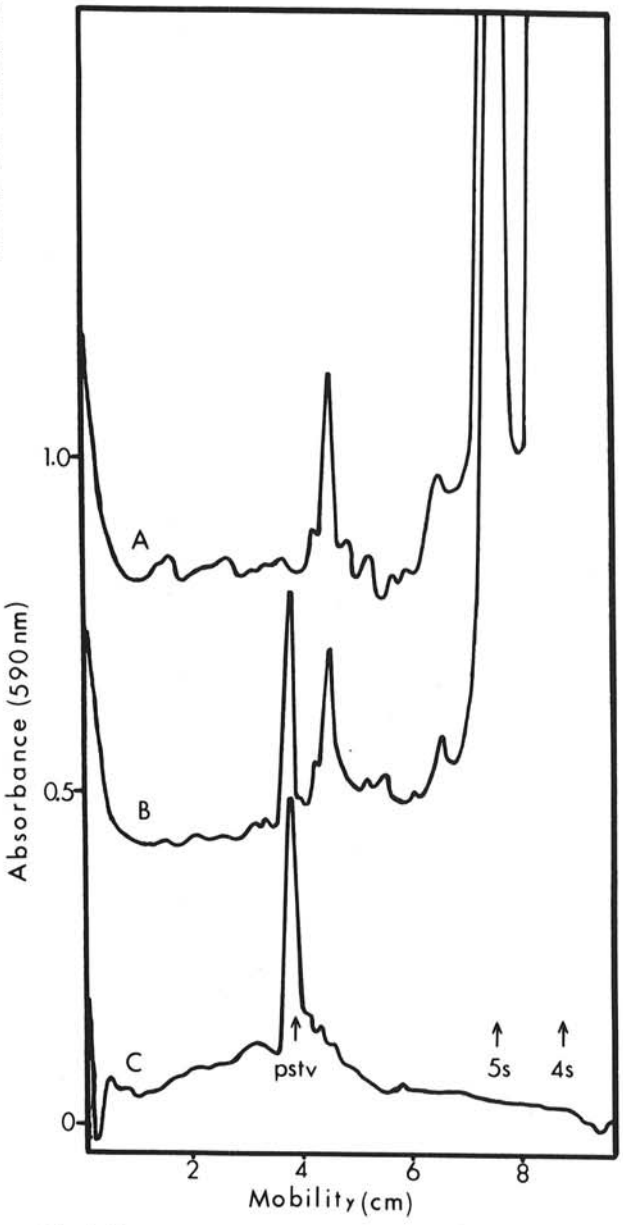

Fig. 5 -(A to C). Scanning profile of stained polyacrylamide gels (5%) containing nucleic acid extracted from potato tissue. The RNA was electrophoresed at 100 V, 6 mA/gel for 4 hours at 12 C and stained in 0.1% toluidine blue O. **A)** healthy plant. **B)** infected plant. **C)** PSTV-RNA purified by gel electrophoresis.

relatively constant in plants grown from infected tubers throughout the experiment. Plants infected with the severe strain of PSTV had a slightly higher concentration of viroid than those infected with mild strain. This result was consistent with the higher total nucleic acid content per gram of tissue in plants infected with the severe strain. The concentration of mild and severe PSTV also was determined in the upper half of inoculated tomato plants at times after inoculation. Potato spindle tuber viroid increased slowly to a detectable level at about 20 days postinoculation (p.i.) and reached a maximum in about 80 days. The amount of PSTV in only the young growing tips of the inoculated tomatoes was about equal to that in young potato tips at about 3 months p.i. (Table 1) and did not change significantly at later sampling dates. It is suggested that the long lag time for PSTV to attain maximum concentration in inoculated plants probably reflects the time it takes for all cells to become infected. A constant amount of viroid RNA is then synthesized once all the cells are infected. This is supported by the observation that plants grown from tubers produced by infected potatoes contain a relatively constant amount of PSTV in actively growing tissue throughout the growing season (T. J. Morris, *unpublished*).

Tomato indexing test.—The following test was developed to supplement the single-plant indexing method using polyacrylamide gels. The gel method is too time-consuming to monitor incidence of PSTV in large planting of elite or foundation seed stock. This method involves an intermediate bioassay on tomato seedlings which are subsequently indexed by the gel method. This intermediate step on tomato allows for detection of 1 infected potato in 250.

A single leaf is removed from each potato plant in the field. Bags of 250 leaves are collected for each sample with precautions taken to avoid cross-contamination between samples. A 0.5-cm diameter leaf disk is removed from each leaf in a sample by stacking the leaves and removing disks with a 0.5-cm diameter cork borer. (Two borings per 250-leaf sample yields 500 disks and improves the efficiency of the test.) The disk sample is then extracted for nucleic acid as described in the gel method. After the initial extraction the nucleic acid is ethanol-precipitated and the precipitate is resuspended in 2 ml of inoculation buffer (0.05 M glycine, 0.30 M K_2HPO_4, pH 9.2). This solution may be stored frozen or used directly to inoculate tomato seedlings (three seedlings per pot, using a cotton

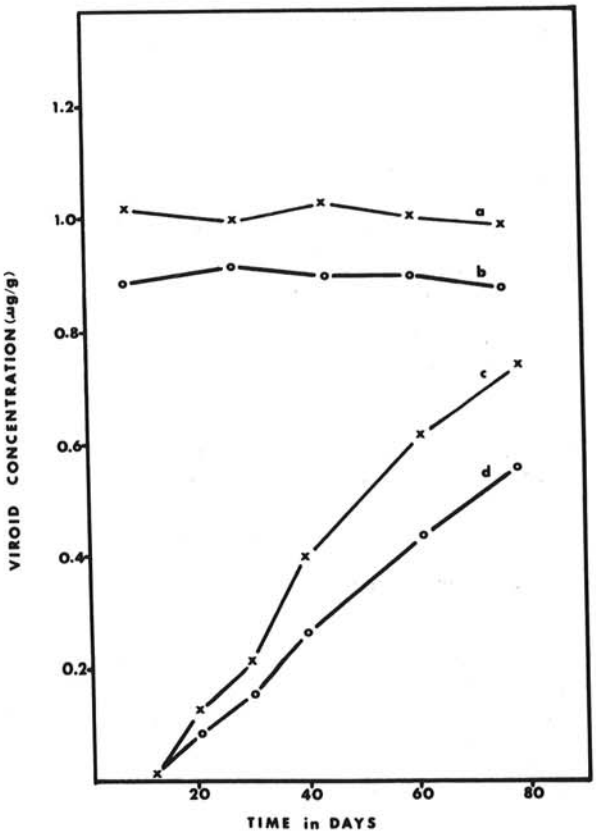

Fig. 6-(A to D). The concentration of potato spindle tuber viroid RNA (micrograms of viroid per gram fresh weight of tissue) in potato tips at times after planting of infected tubers and in the upper half of tomato plants at times after inoculation. **A)** severe and **B)** mild strain in potato. **C)** severe and **D)** mild strain in tomato.

TABLE 1. Effect of temperature on the concentration of mild (M) and severe (S) strains of potato spindle tuber viroid in infected plant tissue

	Concentration of nucleic acid in tissue					
	Growing tip		Mature leaf		Stem	
Sample[a]	Viroid[b]	Total N.A.[c]	Viroid	Total N.A.	Viroid	Total N.A.
Tomato						
M-PSTV, 30 C	1.09	168	0.42	86	0.31	49
S-PSTV, 30 C	1.18	179	0.48	79	0.30	61
S-PSTV, 25 C	0.68	183	0.35	85	0.26	51
Potato						
M-PSTV, 30 C	1.26	191	0.73	131	0.48	98
S-PSTV, 30 C	1.31	198	0.79	142	0.51	91
S-PSTV, 25 C	0.60	189	0.31	153	0.21	79

[a]Tomato plants sampled 80 days postinoculation.
[b]Micrograms of PSTV per gram of tissue determined by planimetry of gel scans taken at 280 nm. Average of three determinations.
[c]Micrograms of LiCl-soluble nucleic acid (N.A.) per gram of tissue as determined by spectroscopy. Average of three determinations.

TABLE 2. Detection of potato spindle tuber viroid (PSTV) in potato using a biological assay on tomato and polyacrylamide gel electrophoretic analysis of nucleic acid extracted from the inoculated tomatoes

Content of sample inoculated to tomato[a]		Presence of PSTV in polyacrylamide gel[b]
Healthy disks	Infected disks	
100	0	0/8
100	1	8/8
250	0	0/4
250	1	7/8
500	0	0/4
500	1	5/12
500	2	12/12

[a]Sample consisted of nucleic acid extracted from the number and type of 0.5-cm diameter leaf disks indicated.

[b]Three seedling tomatoes were inoculated per test. The three terminal shoots were extracted together, 30 days after inoculation and tested by gel electrophoresis. Figures describe number of gels with a PSTV band over the number of tests made.

swab and Celite as an abrasive). The tomatoes are then placed in the greenhouse (30 C is optimal) for 3-4 weeks and then indexed for PSTV using the gel method. Symptoms will be evident only on plants infected with the severe strain.

The procedure has worked successfully using both field- and greenhouse-grown material. Table 2 gives a summary of representative results of a number of experiments. It is apparent that the biological assay to tomato will consistently detect one infected potato plant in 250. Greater assurance in detecting the infected plant is obtained when two disks per leaf are used for each sample. Detection of one infeected plant in 500 was possible but not consistent.

DISCUSSION

The methods described have been adopted for routine detection of PSTV. The gel detection procedure has proven to be useful in indexing for PSTV in new potato introductions and pre-elite seed potatoes. Tubers to be planted in the spring were tested by growing plants from single eyes during the winter and testing the young plants by the gel method. Infected plants could then be discarded before planting. In addition, the gel test provided a rapid confirmatory assay for cases in which inspectors were suspicious of particular plants during the growing season. It allowed for early roguing of infected plants because it can be performed rapidly (in 2 days) and on plants of any age. In contrast, biological assays of severe strain on tomato would take up to 4 weeks for symptom development and up to 8 weeks using a cross-protection test to detect mild strains (4, 5).

A proposed certification scheme would involve testing all pre-elite stock to be planted on the seed farm, in groups of five, using the gel test. This testing could be performed by sprouting single eyes during the winter. Any new stock introduced in subsequent years would also be individually tested. In subsequent years, elite and foundation stock produced from the tested pre-elite stock could be tested in batches of 100 or 250 plants to reinforce the initial testing and assure that reintroduction of PSTV does not occur. Infected batches could be quickly discarded early in the season. Finally, the later stages of foundation stock derived from the tested pre-elite plants could be randomly tested in groups of 100 plants per field to monitor incidence of the pathogen—this final test could be adopted as criteria for certifying the seed potatoes to be PSTV-free. If such a system were adopted for 5-6 years, it might be possible to eliminate PSTV from potato seed farms. Such a scheme is plausible because PSTV spreads slowly. Plants infected late in the season often fail to produce sufficient PSTV in their leaves to be a major source of more infections. They can, however, set tubers which are infected (T. J. Morris, *unpublished*) and therefore seed stock should be tested for several years before being certified free of this disease. It is also possible that similar programs could be useful for monitoring or eliminating other viroid diseases such as those reported in chrysanthemum (3, 7) and citrus (8).

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